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Uptake of the Neurotransmitter Histamine into the Eyes of Larvae of the Barnacle (*Balanus amphitrite*)

ANN E. STUART, HAROLD E. MEKEEL, AND ELIZABETH KEMPTER

Department of Cell and Molecular Physiology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7545

Abstract. The photoreceptors of adult barnacles use histamine as their neurotransmitter and take up ^{3}H-histamine selectively from the extracellular medium. We assayed for the uptake of ^{3}H-histamine into the eyes of the free-swimming (nauplius) and settling (cyprid) larval stages of *Balanus amphitrite*. The extracellular space of nauplii proved permeable to dyes below about 800 molecular weight (MW), indicating that ^{3}H-histamine (MW 111) introduced into seawater would have access to internal structures. ^{3}H-Histamine was taken up into nauplii by a process with a K_D of 0.32 μM. Uptake was antagonized by chlorpromazine, which also blocks uptake of ^{3}H-histamine into adult photoreceptors. In autoradiographs of serial sections of nauplii and cyprids incubated in ^{3}H-histamine, the ocelli and compound eyes were labeled; other structures in the animal were not. No eyes or other structures were labeled with ^{3}H-serotonin, a related amine whose transporter commonly transports histamine as well. These experiments show that a histamine-specific transporter similar to that found in the adult is expressed in all of the eyes of barnacle larvae. In the ocelli, where photoreceptors and pigment cells may be distinguished in the light microscope, label was unexpectedly concentrated far more over the pigment cells than over the photoreceptors.

Introduction

The photoreceptors of a wide spectrum of arthropods, including various insects, crustaceans, and arachnids, synthesize histamine and use it as their neurotransmitter (reviewed by Stuart, 1999). In *Drosophila*, the photoreceptors of the compound eyes of adults are immunolabeled for histamine (Sarthy, 1991; Pollack and Hofbauer, 1991) but the photoreceptors of the third-stage larvae are not, suggesting that these cells use a different transmitter (Pollack and Hofbauer, 1991). Substantiating this difference between adult and larva is the finding that mutant flies lacking the synthetic enzyme for histamine are blind but their larvae are not (Melzig *et al.*, 1996). Indeed, it is likely that the larval photoreceptors employ acetylcholine as their neurotransmitter (Yasuyama *et al.*, 1995). This observation led us to ask whether the histaminergic neurotransmitter system is expressed in the larvae of another arthropod, the barnacle, where the visual system undergoes dramatic changes during the animal's life cycle (Thorson, 1964; Crisp and Ritz, 1973; Lang *et al.*, 1979).

Histaminergic photoreceptors take up ^{3}H-histamine, added to the extracellular medium, *via* a histamine-specific transporter (Stuart *et al.*, 1996; Melzig *et al.*, 1998; Battelle *et al.*, 1999; Borycz and Meinertzhagen, 2000) and are clearly labeled in autoradiographs. In the present study, we used autoradiography as well as biochemistry to assay for the uptake of ^{3}H-histamine into the eyes of barnacle larvae. We find that ^{3}H-histamine is taken up into both the simple and compound eyes but not into other cells in the animal. Thus the larvae have a histamine transporter as does the adult. Unexpectedly, label was more concentrated over the pigment cells surrounding the light-sensitive dendrites of the photoreceptors than over the photoreceptors themselves.

Materials and Methods

Animals and preparations

Nauplius and cyprid larvae of *Balanus amphitrite* were obtained from the laboratory of Dr. Daniel Rittschof at the Duke University Marine Laboratory, Beaufort, North Carolina, where they were collected after induced release from adult animals maintained in the laboratory (Rittschof *et al.*,

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E-mail: stuart@med.unc.edu

1984). Nauplii used in experiments were in the first or second instars of larval development. Larvae were concentrated to approximately 2000–6000 nauplii/ml either by attraction to a spot of light or by cooling to 6°C to inhibit swimming. Nauplii swim actively at room temperature, the temperature at which experiments were carried out.

Assay of ^{3}H-histamine uptake by scintillation counting

Nauplius larvae were incubated in 100 μl of 0.2 μ*M* ^{3}H-histamine (Amersham; 36–55 Ci/mmol) for 15 min in Eppendorf tubes at room temperature in the light, as noted in the Results. At the end of the incubation, 1 ml of 12.5 m*M* Co^{2+}/2.5 m*M* Ca^{2+} saline was added to the tube to minimize release of any ^{3}H-histamine taken up (Stuart *et al.*, 1996). The contents of the tube were then filtered and washed with 10–20 ml of 12.5 m*M* Co^{2+}/2.5 m*M* Ca^{2+} saline using a Millipore 12-unit filtration device (Whatman GF/C filters). Radioactivity on each filter (in the nauplii) was then counted using Biofluor scintillate. In experiments in which ionic content was altered, nauplii were filtered onto Millipore 0.45-μm filter disks and rinsed with 1 ml of the altered ion solution. The disk containing the nauplii was then placed into the incubation saline.

Autoradiography

For autoradiography incubations, larvae were further concentrated as follows. The larvae were pipetted into the front chamber of a Millipore filter holder (modified by sawing off the projecting needle connector) and sucked onto the filter disk (Millipore 0.45 μm) within the unit by an attached syringe. In this fashion the larvae were concentrated onto the filter. This procedure did not kill the larvae; when the disk was then removed and touched to a drop of incubation medium, they swam normally. Larvae were incubated in 100 μl of 20 μ*M* ^{3}H-histamine (Amersham; 36–55 Ci/mmol) or 40 μ*M* ^{3}H-serotonin (Amersham or New England Nuclear; 18.2 or 24.7 Ci/mmol) for 15 min in Eppendorf tubes or in isolated drops on Parafilm at room temperature in the light. Triton X-100 (0.1%) or digitonin (0.25%) were included in incubations of cyprid larvae for autoradiography.

Following incubation, larvae were washed from the final filter disk into a glass scintillation vial and fixed overnight in glutaraldehyde (250 m*M*, E. M. Sciences) fixative modified from Hudspeth and Stuart (1977) to contain 0.2 *M* Na^+ cacodylate (pH 7.7) instead of phosphate buffer. Fixed larvae were postfixed in osmium tetroxide (1%) in cacodylate buffer (1 h, room temp), dehydrated, and immersed in Epon/propylene oxide (1:1) overnight. The vial was left uncapped in the hood and the propylene oxide allowed to evaporate. The larvae were then spread about on an aluminum foil weighing dish and covered with drops of fresh Epon for 1 h for the plastic to infiltrate the tissue. Individual larvae were then lifted from the dish with a sharpened orangewood applicator stick, placed in a new foil dish, covered with a thin layer of Epon, and embedded at 60°C overnight. Selected larvae were oriented as desired by re-embedding in either inverted Beem capsules or latex coffin molds.

Blocks were serially sectioned at 2 μm. Sections were dried on gelatin-coated slides, dipped in Kodak NTB-2 autoradiographic emulsion, exposed at 4°C for 5 days to 2 weeks, and developed in D-19 (Kodak).

Dye exclusion assays

Fluorescent dye exclusion assays were performed by placing live nauplii in saline containing 2% Lucifer yellow CH, Procion yellow MX-4R, Procion yellow H-5G, or Procion yellow H-5G covalently bound to a dextran of about 40,000 MW (all Sigma). Following 15 min in dye, the nauplii were filtered, suspended in O.C.T. compound mounting medium (Pelco International), placed in a plastic capsule, and immediately frozen in a beaker of isopentane (Kodak) precooled by dry ice. Nauplii were then sectioned at 10 μm on a cryostat, placed on Fisher Superfrost-plus charged slides, coverslipped with 2 drops of glycerol/N-propyl gallate, and viewed under a fluorescence microscope with XF22 filters (Omega).

Results

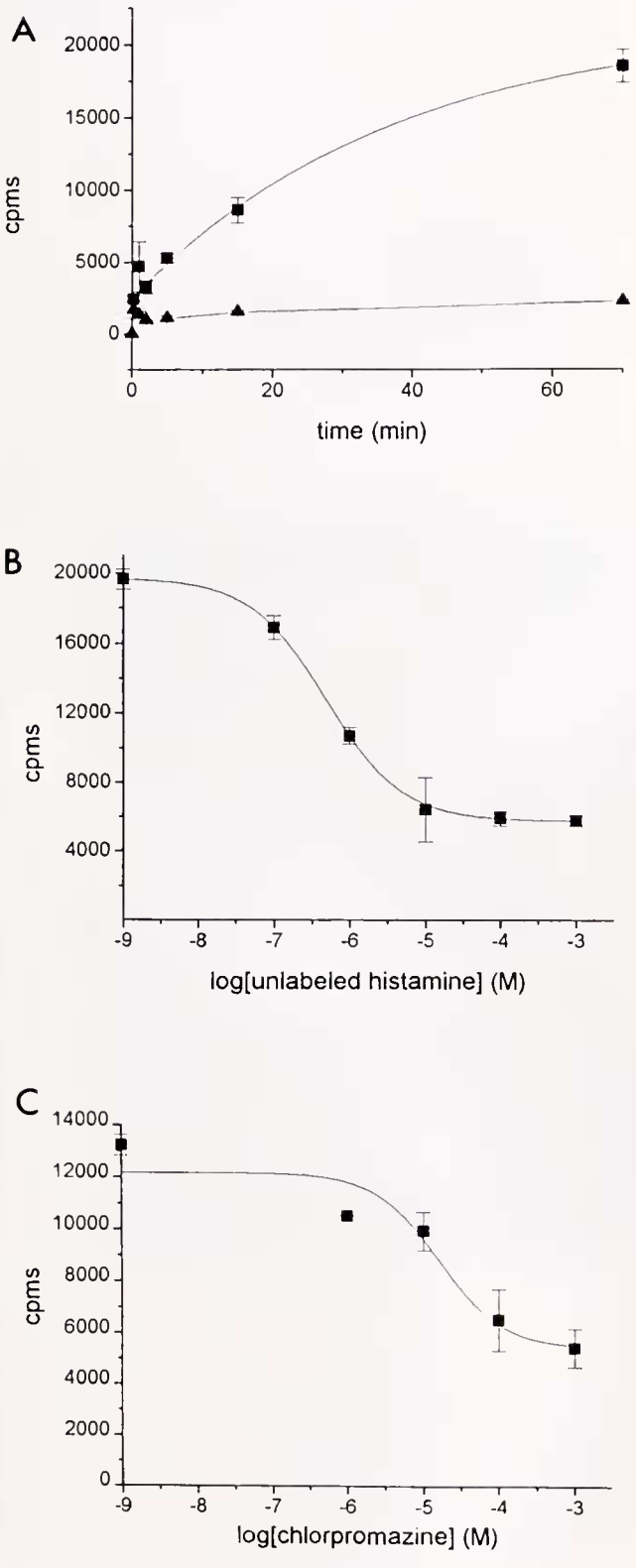
Barnacles (of the genus *Balanus*) have three stages to their life cycle: the free-swimming nauplius; the cyprid, specialized for settling; and the reproductive adult. The nauplius has one simple eye and swims towards light, presumably to disperse (Thorson, 1964; Sastry, 1983). The cyprid retains the simple eye but, in addition, suddenly develops a pair of compound eyes (Barnett *et al.*, 1979), presumably to guide the animal in settling appropriately. The sessile adult sheds the compound eyes (Walker and Lee, 1976), no longer needed, and retains the simple eye, which separates into the median and paired lateral eyes of the adult (Takenaka *et al.*, 1993) that mediate a defensive response to shadows.

Penetrability of larvae

The epidermis of early-stage nauplii is not well developed (Freeman, 1993), suggesting that substances introduced into seawater might diffuse into the animal's extracellular space and be accessible to internal structures such as the ocelli. To test the permeability of nauplii, we exposed them to dyes ranging in molecular weight from 289 to 40,000.

Simply observing living, dye-soaked nauplii through the light microscope suggested that lower molecular weight dyes penetrated into the animals, whereas a dye of higher molecular weight did not. The cutoff appeared to be at a

molecular weight of about 800: neutral red (MW 289), toluidine blue (MW 306), neutral violet (MW 409), and ruthenium red (MW 786) stained the animal, whereas fast green (MW 809) did not. To make certain that the dyes were indeed inside the animal, nauplii were exposed to four fluorescent dyes of differing molecular weights, frozen quickly, and sectioned on a cryostat. Fluorescence was found throughout the sections of animals exposed to Lucifer yellow CH (MW 457.2) and Procion yellow MX-4R (Reactive orange 14) (MW 631.4), but not in sections of animals exposed to Procion yellow H-5G (Reactive yellow 2) (MW 873) or its dextran-bound derivative (MW ~40,000). We conclude that the naupliar epidermis does not present a significant barrier to compounds of molecular weight below about 800 and that substances such as ^{3}H-histamine (MW 111) might be expected to penetrate easily into the animal and be taken up by internal structures.



Biochemistry

When incubated with ^{3}H-histamine (0.2 μM), nauplii took up this compound in a time-dependent fashion (Fig. 1A). The time course of uptake could be fitted with an exponential association curve with a rate constant of 0.028/min. Inclusion of an excess (2 mM) of unlabeled histamine in the incubation medium blocked the uptake of labeled compound (Fig. 1A), indicating that uptake is a competitive process. From the dose/response curve describing this competition (Fig. 1B), a K_D of 0.32 μM was calculated for ^{3}H-histamine uptake, a value suggesting a high-affinity uptake mechanism.

Chlorpromazine, which antagonizes the uptake of ^{3}H-histamine into the photoreceptors of adult barnacles (Stuart *et al.*, 1996) and into snail neurons (Osborne *et al.*, 1979), also markedly decreased the uptake of ^{3}H-histamine into nauplii (Fig. 1C) (K_i = 21 μM). Block appeared to be incomplete, as is the case with adult photoreceptors (Stuart *et al.*, 1996), although block of uptake of ^{3}H-histamine by a high concentration of drug was not compared with competitive antagonism by cold histamine in the same assay. The maximum inhibition in the adult was about 70% (Stuart *et al.*, 1996) and in nauplii about 50%. The sensitivity of ^{3}H-histamine uptake to chlorpromazine in both the larvae and the adult suggests that it is mediated by a similar

Figure 1. Uptake of ^{3}H-histamine into nauplius larvae assayed by scintillation counting. (A) Time course of uptake. Two equal aliquots of larvae were incubated for each time point (0,1,5,15, or 70 min) in 0.2 μM ^{3}H-histamine without (■) or with (▲) 2 mM unlabeled histamine. Addition of unlabeled histamine to the incubation medium blocks the uptake of ^{3}H-histamine. (B) Competition of unlabeled histamine with ^{3}H-histamine for uptake into nauplii. The data were fit to a one-site competition algorithm with a K_D of 0.32 μM. (C) Chlorpromazine antagonizes the uptake of ^{3}H-histamine into nauplii. Aliquots of larvae were incubated with increasing concentrations of drug, as described in Materials and Methods. A simple competition fit to the points gives a K_i of 21 μM. In each experiment the symbol shows the average of the values for two samples, and the bar shows the range of these values; cpms = counts per minute.

transporter. Nauplii incubated either in 2 m*M* histamine or in 20 μM chlorpromazine (which would presumably have increased extracellular histamine by blocking uptake) stopped swimming towards the light and eventually sank to the bottom of the tube. The current notion of histamine's action in the visual pathway suggests that the presence of histamine in the cleft would lead to more movement, not less (see Discussion); thus these observations cannot easily be explained and may involve histamine's actions on neurons other than those of the visual system in the larva.

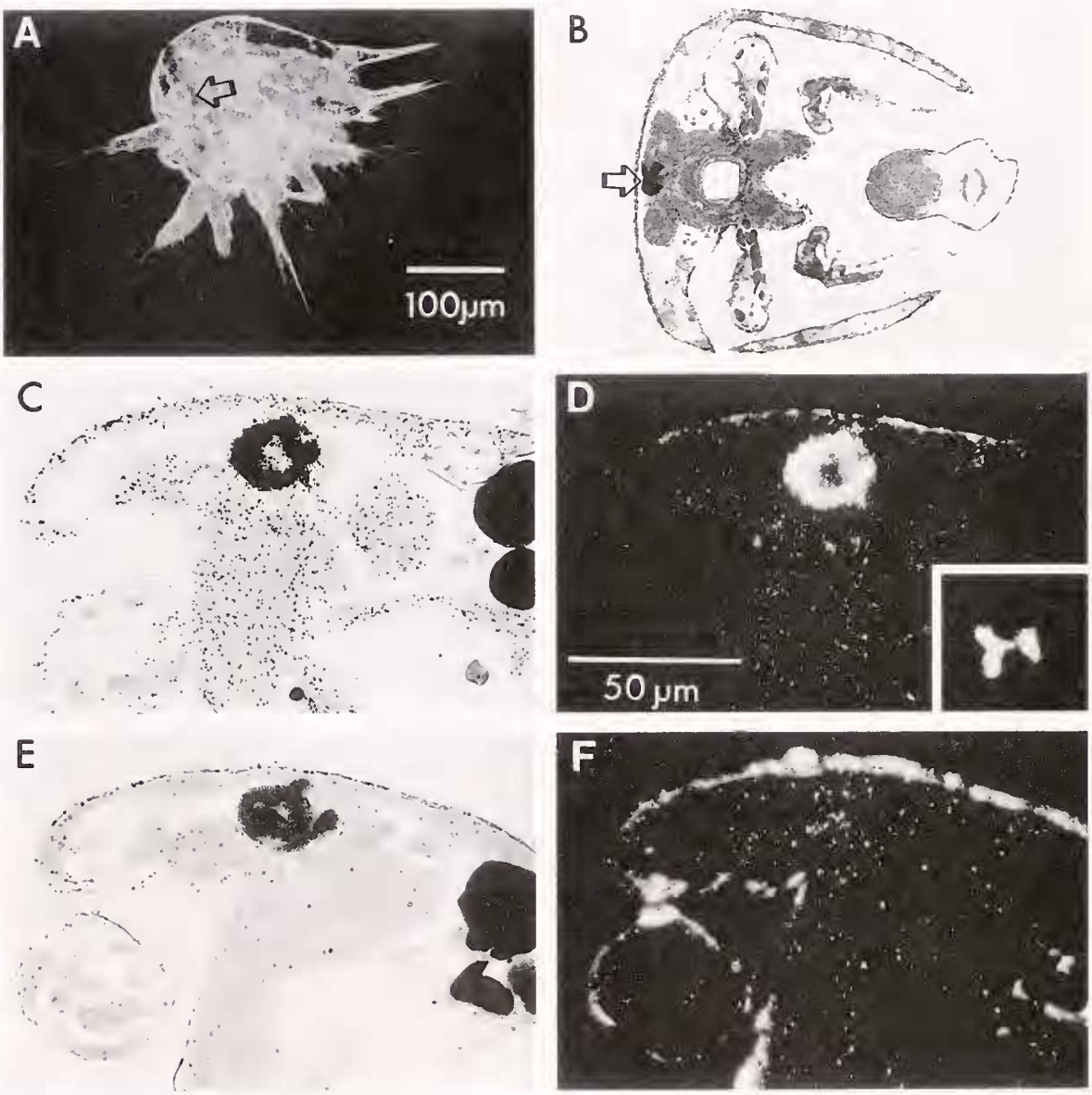


Figure 2. Uptake of ^{3}H-histamine into the ocellus of nauplius larvae as revealed by autoradiography. (A) A whole mount of a nauplius larva viewed from the side. (B) A section of a nauplius larva through the ocellus, viewed from the top. The pigmented ocellus (arrows in A and B) is obvious within this transparent animal and in sections. (C) Brightfield and (D) epipolarization illumination of an ocellus in a 2-μm section of a nauplius that had been incubated in ^{3}H-histamine. Accumulation of silver grains over the ocellus cannot be distinguished from pigment in brightfield illumination but is obvious in epipolarized light, which reflects from the silver grains but not from the pigment. The insert shows a section through another eye at a different orientation in which the pigment arms form two back-to-back cups around the photoreceptors; the right arm of the upper cup is incomplete. (E) Brightfield and (F) epipolarization illumination of a 2-μm section of a nauplius that had been incubated in ^{3}H-serotonin. There is no accumulation of grains over the ocellus. Scale bar in D applies to C–F.

Autoradiography of nauplius larvae

The single, median eye (the ocellus) of the nauplius (Fig. 2A, B, arrows) is a striking feature of this larval stage. The two pigment cells at the center of this simple eye give it a deep red color that allows it to be clearly seen, even under the dissecting microscope. The photoreceptors are subdivided by these pigment cells into three groups that "look" in three different directions. The pigment cells are shaped to form three cups, and within each cup are two to six photoreceptors, termed retinular cells (Kauri, 1962; Walley, 1969; Walker *et al.*, 1987; Clare and Walker, 1989). A comparison of the light intensity on each group of photoreceptors, made by higher-order cells, is presumably the mechanism that permits these free-swimming stages to determine from which direction the light is coming.

In sections viewed at high power with the light microscope, the pigment cells may be clearly differentiated from the photoreceptors enveloped by their processes (*e.g.*, Fig. 3B). A pigment cell cup may have been sectioned horizontally across the cup wall so that the pigment forms a ring (Fig. 2C, D). Alternatively, the section may reveal the cuplike shape of the cell (Fig. 3) as it envelopes the photoreceptor processes. The long, dark lines of the rhabdomes of the apposed photoreceptor neurons (Takenaka *et al.*, 1993) may be seen especially well in Figure 3B, forming a "V" within the arms of the pigment cells.

To determine whether the ocellus or other structures within the nauplius larva take up ^{3}H-histamine, serial sections through nauplii incubated in ^{3}H-histamine (20 μM) were exposed for autoradiography. Examination of these sections revealed that the label is concentrated over the ocellus but not over other parts of the animal (Fig. 2D). The ocelli of larvae incubated in an excess of unlabeled histamine (2 mM) added to the ^{3}H-histamine were not labeled above background (not shown). Unexpectedly, the regions within the pigment ring or cup, where the photoreceptors lie, are labeled only weakly compared to the labeling of the pigment cells (Figs. 2C, D; 3A). Brightfield photographs (Fig. 2C) do not allow one easily to distinguish silver grains from pigment, but in epipolarized light (Fig. 2D) the pigment does not reflect the light and the silver grains stand out clearly. The inset of Figure 2D shows a section through another eye in which two back-to-back, labeled pigment cell cups form an incomplete "H" as they extend arms around two groups of photoreceptors (Takenaka *et al.*, 1993).

Nauplii incubated in ^{3}H-serotonin (20 μM) did not show increased labeling over the ocellus or, indeed, over any structure (Fig. 2E, F; Fig. 3B). Adult *Balanus nubilus* photoreceptors are not labeled when incubated with ^{3}H-serotonin, but a small number of other neurons within the adult nervous system do show labeling (Stuart *et al.*, 1996). Further, serotonin has been detected in cyprid larvae and appears to be important in settlement (Yamamoto *et al.*,

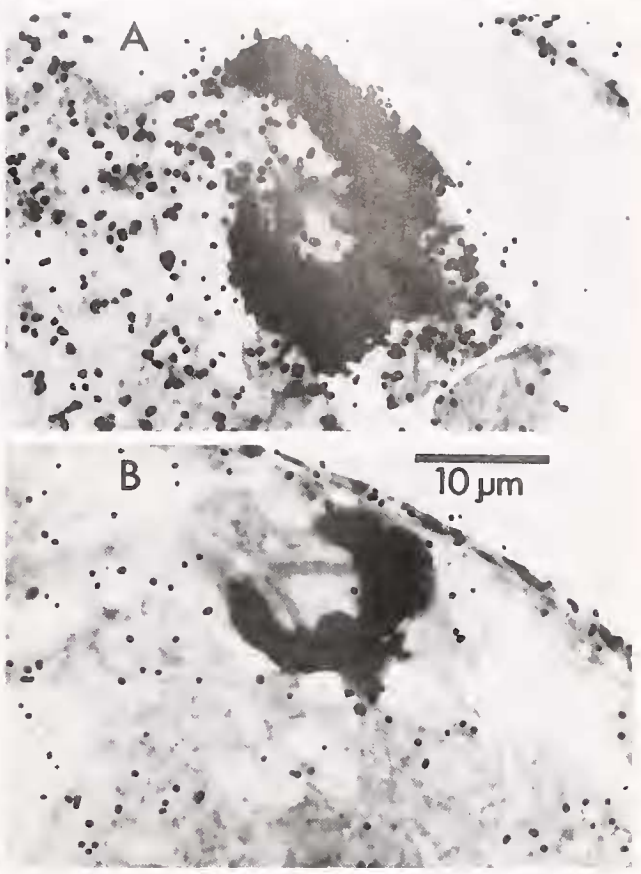


Figure 3. Autoradiographs of sections through ocelli of nauplii that had been incubated in (A) ^{3}H-histamine or (B) ^{3}H-serotonin. In both autoradiographs, the rhabdomes of the photoreceptors are visible as two gray lines forming a "V" (pointing to the upper left) within the arms of the pigment cells. In (A) grains are concentrated over the pigment cells (same animal as in Fig. 2C, D). Although the grains are difficult to distinguish from pigment in brightfield illumination, they clearly are not concentrated over the photoreceptors, whose processes lie within the pigment cell cup. In (B) there is no concentration of grains over either cell type (same animal as in Fig. 2E, F).

1999). In the nauplius larva these neurons may not have developed as yet or may have been too small to detect, or their absence may reflect a species difference between the adult *Balanus nubilus* and the larval *Balanus amphitrite* nervous systems. The lack of labeling of the ocelli with ^{3}H-serotonin indicates that the uptake of ^{3}H-histamine into barnacle eyes is by a process selective for histamine in the nauplius larva, as it is in the adult.

Autoradiography of cyprid larvae

A whole mount of a cyprid larva viewed from the side is depicted in Figure 4A, and a section through the eyes of a cyprid viewed from the top is seen in Figure 4B. The medially located ocellus (smaller arrows) and laterally located pair of compound eyes (larger arrows) may be clearly seen. Cyprids were incubated in ^{3}H-histamine (20 μM) and

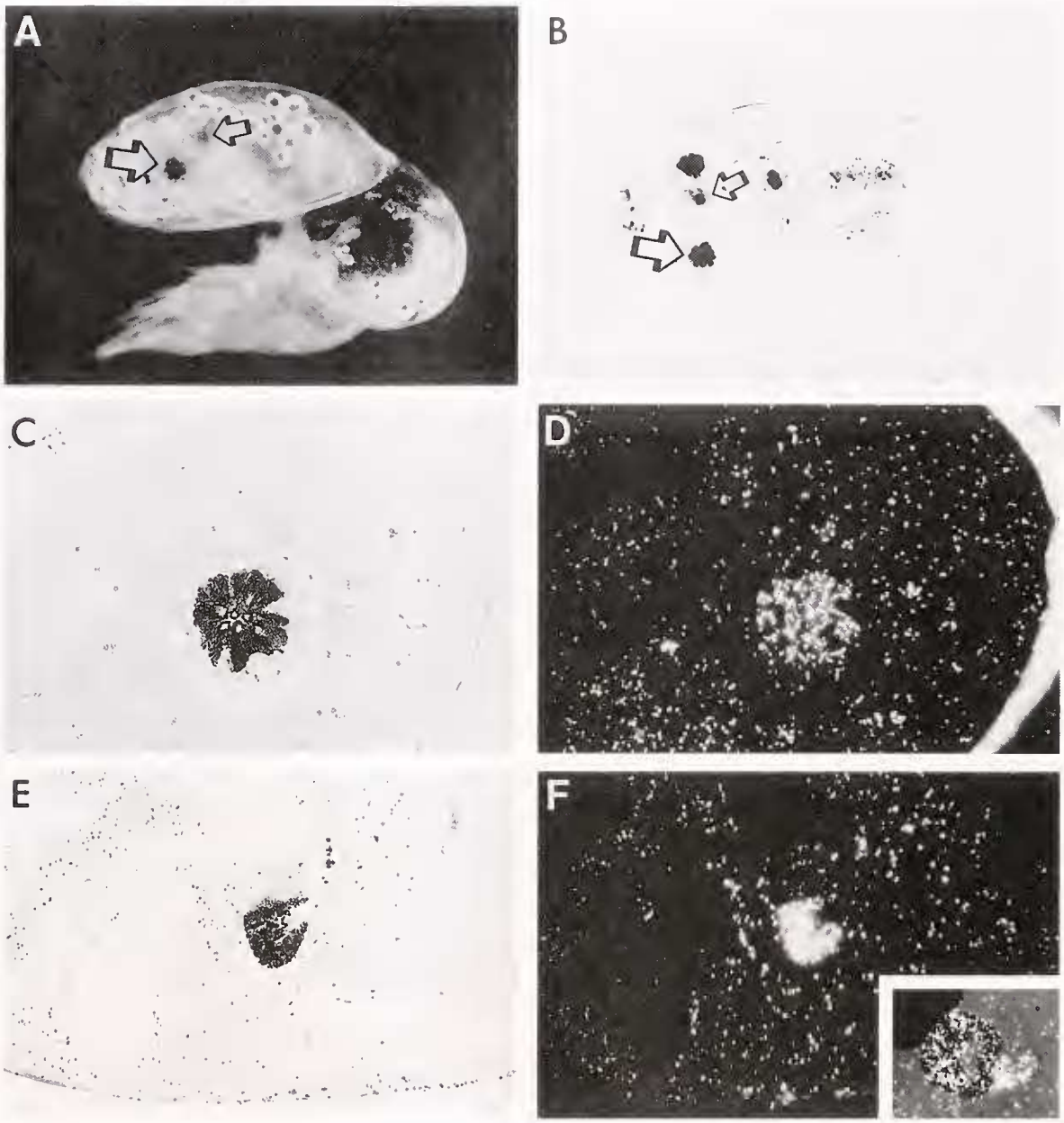


Figure 4. Uptake of ^{3}H-histamine into the compound eyes and ocellus of the cyprid larva as revealed by autoradiography. (A) Whole mount of a cyprid larva viewed from the side, and (B) a section through the three eyes of a cyprid viewed from the top. Compound eyes are labeled with larger arrows and the ocellus with a smaller arrow in A and B. (C) Brightfield and (D) epipolarization illumination of a compound eye in a 2-μm section of a cyprid that had been incubated in ^{3}H-histamine and Triton X-100, showing increased label over the eye compared to surrounding tissues. (E) Brightfield and (F) epipolarization illumination of the ocellus in the same animal shows a pattern of label over the pigment cells. (Inset) Phase contrast/epipolarization illumination of the ocellus from another animal.

processed for autoradiography. Unlike nauplii, cyprids have a thick cuticle overlying the epidermis, so would be expected to be poorly penetrated by substances added to the seawater. Indeed, when the larvae were incubated in ^{3}H-histamine alone, the eyes were labeled only faintly. Addition of the solubilizing agent Triton X-100 during the incubation increased the degree of labeling of both the compound eyes (Fig. 4C, D) and the median eye (Fig. 4E, F). The cuplike shape of the label within the median eye (Fig. 4E, F) again indicates that the pigment cells may be more heavily labeled than the photoreceptors. A section through the eye of another specimen, catching the pigment

cup as a ring, indicates that grains might also be concentrated within and beside the cup (Fig. 4F, inset). It was not possible to determine which structures were labeled within the compound eye.

Discussion

We report here that all of the eyes of both the nauplius and cyprid larvae of balanoid barnacles are labeled when incubated in ^{3}H-histamine, but that other tissues in the animal are not. Uptake is blocked by chlorpromazine and is specific in that ^{3}H-serotonin is not taken up, as is also the case with uptake of ^{3}H-histamine into the adult median photoreceptors. Thus a histamine transporter similar to that of the adult appears to be expressed in the eyes of the larvae.

In the simple median eyes of both the nauplii and cyprid, autoradiographs showed label concentrated primarily over the pigment cells surrounding the photoreceptors rather than over the photoreceptor rhabdomeric processes enveloped by the pigment cells. But histamine uptake may take place only in the axons and terminals of these sensory neurons, as it does in the adult (Stuart *et al.*, 1996). In the larvae, the axons of the photoreceptors could not be reliably followed to settle this point. We also do not know if the heavy labeling of the pigment cells is specific to larvae, since in the adult there are no pigment cells associated with the commonly studied median eye. Adult lateral eyes of certain species retain pigment cells, but the uptake of ^{3}H-histamine has not been studied in those cases. It is not yet possible, then, to determine whether ^{3}H-histamine uptake in the larval eyes follows or differs from the adult pattern.

Although the behaviors of the nauplius larva and the adult barnacle with respect to light seem very different at first glance, they are actually similar and indeed might not require radical changes in circuitry (such as a different photoreceptor transmitter) during development. In both larvae and adults, changes in the ambient light affect the rhythmic motion of the animal's appendages, the cirri. In the larvae, this motion underlies swimming, whereas in the adult it is devoted to feeding.

In adult barnacles, rhythmic feeding by the cirri proceeds in the light, but the dimming of light halts rhythmic cirral motion and provokes the retraction of the cirri into the shell. The visual pathway is kept from interfering with the cirral rhythm as long as the light is not disturbed: histamine, continuously liberated by the illuminated photoreceptors, inhibits the postsynaptic cells, keeping the visual pathway silent. Dimming of the light (for example, by shadows of predators) interrupts the release of histamine, releases the second-order cells from inhibition, and initiates impulses in the visual pathway (Stuart and Oertel, 1978), leading to the cessation of these feeding movements. A complex behavior involving most of the animal's muscles is set in motion: the cirri stop their rhythmic motion, withdrawing into the shell, and the opercular plates are pulled closed to seal the animal within its fortress (Gwilliam, 1963).

In the nauplius larvae, the job of the cirri is to propel the animals toward light. The direction from which the light is coming is determined by the ocellus. The three divisions of the naupliar eye look in three different directions, and presumably the division in greatest light somehow causes the strongest movement of the animal in that particular direction. Thus the function of light in the nauplius and adult would seem similar in that light is accompanied by movement (feeding in the adult, swimming in the nauplius) and dimming or less light by the cessation or a decrease of movement. It might be that the neural circuits are similar at the two stages, although they accomplish very different behaviors depending on whether the animal is free-swimming or sessile.

Acknowledgments

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